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## **Imiquimod Induce CCR9 Ameliorates Murine TNBS Colitis**

Running title: TLR7 inducing Tregs ameliorate experimental colitis

Ryoma Suzuki, Kyoko Katakura\*, Tatsuo Fujiwara, Naohiko Gunji,  
Hiroshi Watanabe, and Hiromasa Ohira

Department of Gastroenterology and Rheumatology, Division of Medicine,  
Fukushima Medical University, School of Medicine, Fukushima, 960-1295, Japan

**Specific author contributions:** Study design: KK; research and data analysis: RS, KK, TF and NG; manuscript drafting: KK and HO; critical revision: HW

\*Correspondence to: Kyoko Katakura MD., Ph.D., Assistant Professor,

Department of Gastroenterology and Rheumatology, Division of Medicine, Fukushima

Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan. [k-kata@fmu.ac.jp](mailto:k-kata@fmu.ac.jp)

Tel: +81-24-547-1202, FAX: +81-24-547-2055,

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## **Abstract**

**AIMS:** To investigate whether IMQ as TLR7 ligand protects mice from colonic inflammation and the mechanisms underlying in such immunoregulatory conditions.

**METHODS:** Murine colitis was induced to Balb/c mice by administration of trinitrobenzene sulfonic acid (TNBS) with or without daily intraperitoneal administration of IMQ. Colitis was evaluated by body weight decreases and by histological score. Also colonic mRNA expression was measured by RT-PCR. To confirm the induction of Regulatory T cells (Tregs) by type-1 IFN from pDCs, we generated mouse bone marrow-derived pDCs and co-cultured these with CD4<sup>+</sup> T cells isolated from mouse spleen with or without IMQ stimulation. Cytokine production in the culture supernatant was measured by ELISA and number of Tregs were analyzed by flow cytometry. Spleen and mesenteric lymph nodes (MLN) from IMQ-treated mice were collected, and mRNA expression of cytokine was measured by RT-PCR and cytokine production were measured by ELISA. Tregs and chemokine expression were analyzed in TNBS colon by immunohistochemistry. **RESULTS:** Administration of IMQ significantly suppressed colonic inflammation of TNBS-induced colitis. In the colons of IMQ-treated mice, mRNA expression of TNF- $\alpha$  was decreased, and strong expression of IL-6, IFN- $\beta$  and TGF- $\beta$  was detected. IL-10 and TGF- $\beta$  production was increased in the supernatant of co-cultured cells stimulated with IMQ, although we were unable to detect Treg induction in IMQ-stimulated co-cultured cells. In MLN of IMQ-treated mice, strong expression of TLR7, IFN- $\beta$ , TGF- $\beta$  and Foxp3 mRNA was detected. IL-10 production from MLN cells was also increased in the IMQ-treated

group. Finally, Tregs in the inflamed colon and CCR9 in MLN of IMQ-treated mice were detected. **CONCLUSION:** These results suggest that IMQ protects mice from TNBS colitis through induction of CCR9, which regulates accumulation of Tregs in the inflamed colon.



## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel disease (IBD) with unknown etiology. The current hypothesis states that IBD results from an inappropriately elevated immune response to resident intestinal bacteria<sup>1</sup>. The intestinal mucosa is normally maintained with protective immunity and tolerance to self-antigens and commensal bacteria<sup>2</sup>. It has been showed that colonic homeostasis is maintained by innate immune systems mainly via Toll-like receptor (TLR) signaling<sup>3,4</sup>. In addition to innate immunity, peripheral tolerance is maintained by regulatory T cells (Tregs), a population of CD4<sup>+</sup> T cells that regulates the proliferation and effector functions of other T cells<sup>5</sup>. There is growing evidence that Th17-cell-driven immune response contributes to the pathology of IBD<sup>6,7</sup>, and establishing a balance between Tregs and Th17 cells may therefore be effective in the treatment of IBD.

Dendritic cells (DCs) are the only cells able to stimulate naïve T cells and are therefore considered to be the main antigen presenting cells (APCs) for T lymphocytes<sup>8</sup>. Functionally different subsets of DCs have been described. So-called conventional DCs (cDCs) can be separated in lymphoid organ-resident DC subsets and peripheral tissue DCs (Langerhans cells and interstitial DCs), which can migrate to lymph nodes, but not the spleen<sup>9</sup>. Plasmacytoid DC (pDCs), also known as interferon (IFN)-producing cells, are found in blood and secondary lymphoid organs, but not peripheral tissue in the steady state<sup>10</sup>. cDCs express a large repertoire of Toll-like receptor (TLR), whereas pDCs selectively express TLR7 and TLR9. In the steady state in vivo, the phenotype of pDCs is immature and their role in T cell stimulation remains controversial<sup>11</sup>. However,

pDCs can be recruited to inflamed lymph nodes and once mature, pDCs can efficiently stimulate naïve T cells<sup>12</sup>. Also in previous report, chemokine receptor CCR9 was induced by TLR9 activation<sup>13</sup>. CCR9 is a key regulator of leukocyte migration and CCR9<sup>+</sup>pDCs are potent inducers of Tregs<sup>14</sup>.

We have been demonstrated that TLR signaling in the intestine, mainly TLR9 inhibits inflammatory responses via the induction of type-1 IFN in the mouse colitis model<sup>15</sup>. Imiquimod (IMQ) is a small-molecule immunomodulatory compound of the imidazoquinoline family that exhibits both antiviral and antitumor properties<sup>16</sup>. As a nucleoside analog that mimics the immune response to viral ssRNA, IMQ exerts biological efficacy through agonistic stimulation of TLR7 in immune cells, resulting in the production of cytokines and chemokines similarly to TLR9<sup>17, 18</sup>. Commercially, IMQ has been effectively used as a topical agent for therapeutic treatment of a number of dermatological tumors, including basal cell carcinomas<sup>19, 20</sup>, intraepidermal keratinocyte neoplasias<sup>21</sup>, and cutaneous metastases of melanoma<sup>22</sup>.

Here, we report that IMQ protects against mice colitis through induction of chemokines that regulate accumulation of Tregs into the inflamed colon. Our results also suggest that IMQ offers a novel tool for the treatment of human IBD.

## **MATERIALS AND METHODS**

### **Reagents**

The following materials were obtained from commercial sources: 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma Chemical Co., Solon, OH); Purified LPS and Imiquimod (IMQ; InvivoGen, San Diego, CA); and CpG oligodeoxynucleotides (ODNs 1018; Trilink, San Diego, CA)

### **Mice**

Seven- to nine-week-old female Balb/c mice were purchased from CLEA-Japan. Animals were housed under specific pathogen-free conditions. All experimental procedures were approved by the institutional committee for animal care and use of Fukushima Medical University.

### **Induction and evaluation of experimental colitis**

TNBS colitis was induced in mice by rectal instillation of 0.5 mg/mouse of TNBS dissolved in 0.1ml of 50% ethanol<sup>23</sup>. Mice were then weighed and injected with sterile saline or IMQ at a dose of 10 µg/body intraperitoneally for 5 consecutive days. Five days after induction of colitis by TNBS, mice were sacrificed, and entire colons were removed, and length and weight were measured.

### **Histological scoring**

After 5 days of TNBS administration, mice were sacrificed and the entire colon was

excised, opened longitudinally, rolled onto a wooden stick, fixed with 10% Formaldehyde Solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) and embedded in paraffin. Tissue sections were prepared, deparaffinized and stained with hematoxylin and eosin. Histological scores were assigned by experimenters blinded to sample identity. Colonic epithelial damage was scored as follows: 0 = normal; 1 = hyperproliferation, irregular crypts, and goblet cell loss; 2 = mild to moderate crypt loss (10-50%); 3= severe crypt loss (50-90%); 4 = complete crypt loss, surface epithelium intact; 5 = small- to medium-sized ulcers (<10 crypt widths); 6 = larger ulcers ( $\geq$ 10 crypt widths). Infiltration with inflammatory cells was assigned scores separately for mucosa (0 = normal, 1 = mild, 2 = moderate, 3 = severe), submucosa (0 = normal, 1 = mild to moderate, 2 = severe) and muscle/serosa (0 = normal, 1 = moderate to severe). Scores for epithelial damage and inflammatory cell infiltration were added, resulting in a total scoring range of 0-12<sup>15</sup>.

### **Quantitative Reverse Transcriptase - Polymerase Chain Reaction (qRT-PCR)**

Total RNAs were isolated from 100  $\mu$ g of each colonic sections, spleen, and MLN of mice from TNBS-induced colitis. RNA was reverse transcribed to single-stranded cDNA using the Random Primer, dNTP Mixture (TAKARA SHUZO Co., Ltd., Shiga, Japan), and RNasin<sup>®</sup> Ribonuclease Inhibitor (Promega, Madison, WI) according to the manufacturer's protocol. cDNA was used for quantitative analysis by PCR. The following sets were used as PCR primers:

TNF- $\alpha$             (sense)            5'-GCCACCACGCTCTTCTGTCT-3'            (antisense)

5'-GGTCTGGGCCATAGAACTGATG-3';

IL-6 (sense) 5'-ATGAAGTTCCTCTCTGCAAGAGACT-3' (antisense) 5'-  
CACTAGGTTTGCCGAGTAGATCTC -3';

IFN- $\beta$  (sense) 5'-ATGAACTCCACCAGCAGACAG-3' (antisense)  
5'-ACCACCATCCAGGCGTAGC-3';

TGF- $\beta$  (sense) 5'-GATACCAACTATTGCTTCAGCTCCACA-3' (antisense)  
5'-TCAGCTGCACTTGCAGGAGCGCACAAT-3';

IL12-p40 (sense) 5'-AAACCAGACCCGCCCAAGAAC-3' (antisense)  
5'-AAAAAGCCAACCAAGCAGAAGACAG-3';

TLR7 (sense) 5'-TCTTACCCTTACCATCAACCACA-3' (antisense)  
5'-CCCCAGTAGAACAGGTACACA-3';

and GAPDH (sense) 5'-CCAGTATGACTCCACGACATACTCA-3' (antisense)  
5'-ATCAACGACCCCTTCATTGACC-3'.

Quantitative real-time PCR (qPCR) was performed using a StepOne Real Time PCR System (Applied Biosystems, Foster City, CA) with Fast SYBR<sup>®</sup> Green Master Mix (Applied Biosystems). PCR mixtures contained 0.5  $\mu$ M sense and antisense primers. Samples were denatured at 95°C for 20 seconds, followed by 40 cycles of annealing and extension at 95°C for 3 seconds, and 60°C for 30 seconds. Melting curves were obtained at the end of amplification by cooling the samples to 55°C for 15 seconds, followed by further cooling to 60°C for 1 minute and 95°C for 15 seconds. In qRT-PCR, data were normalized against GAPDH.

## **Cell cultures**

### *Dendritic cells*

Bone marrow-derived pDCs were cultured from the long bones of mice in the presence of 100 ng/ml of murine Flt3 ligand (PeproTech Inc., Rocky Hill, NJ) as described previously<sup>24</sup>. RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (JRH Bioscience, Lenexa, KS), 2-ME (100  $\mu$ M), penicillin (5 U/ml) and streptomycin (50 ng/ml) was used as culture medium. Generated pDC were either used immature or pre-stimulated for 24 hours at  $5 \times 10^5$  cells/ml in the presence of LPS (0.5  $\mu$ g/ml), IMQ (100ng/ml) or CpG (10nM).

### *T cells*

Single-cell suspensions of MLN and spleen were prepared by gently teasing in RPMI 1640 medium. Cells were mashed through a 40- $\mu$ m nylon cell strainer (BD Bioscience, San Jose, CA). Subsequently, CD4<sup>+</sup> T cells were isolated using a CD4<sup>+</sup> T cell isolation kit with magnetic particles, in accordance with the manufacturer's instructions (Miltenyi Biotec, Bergish Glndbach, Germany).

### *Treg induction*

Splenic naïve CD4<sup>+</sup> T cells were incubated with unstimulated pDCs at a 5:1 ratio in 6-well plates in the presence of LPS, IMQ or CpG 1018 in complete medium. As controls, naïve CD4<sup>+</sup> T cells were co-cultured with un-stimulated pDCs or pre-stimulated pDCs to except cell-cell interaction. After 48 hours, primed CD4<sup>+</sup> T cells were harvested, and were analyzed for cell surface phenotypes and cytokine production profile.

#### *MLN CD4<sup>+</sup> T cells cytokine production*

MLNs were removed from TNBS colitis mice with or without IMQ treatment. CD4<sup>+</sup> T cells were isolated from MLNs as above, and were re-stimulated at  $2 \times 10^6$  cells/ml in RPMI1640 in plates coated with immobilized anti-CD3 Ab (5 $\mu$ g/ml; BD Phrmingen, Franklin Lakes, NJ) and soluble anti-CD28 Ab (1 $\mu$ g/ml; BD Phrmingen) for 48 hours. Culture supernatants were collected and cytokine profiles of primed T cells were assayed.

#### **Determination of cytokine concentrations**

Production of murine IFN- $\gamma$ , IL-10, TNF- $\alpha$ , IL-4 and IL-17 in culture supernatants were assessed by ELISA using ELISA kits (e-Biosciences, San Diego, CA) according to the manufacturer's instructions.

#### **Flow cytometry**

Co-cultured cells were stained using FITC-conjugated mouse anti-CD4 and APC-conjugated mouse anti-CD25 antibodies (eBioscience) according to the manufacturer's instruction. Surface labeling was followed by permeabilization with the Foxp3 fix/perm solution (eBioscience) and intracellular labeling with a PE conjugated anti-Foxp3 antibody, according to the eBioscience Foxp3 staining protocol. Measurements were performed on a BD FACScan, and were analyzed with Cell Quest Pro analysis software (BD Bioscience).

### **Immunohistochemistry**

Surface antigens were detected using the streptavidin-biotin-labeling immunoperoxidase-staining technique. Colons were removed, placed in compound-embedding medium (OCT; SAKURA, Tokyo, Japan), snap frozen using dry ice, and stored at -80°C. Ten-micrometer sections were cut, collected on poly (L-lysine)-coated slides, and allowed to air dry. Slides were stored at -20°C over desiccant before staining. Before immunostaining, sections were fixed with cold ethanol (15 minutes) and blocked with 1% H<sub>2</sub>O<sub>2</sub> (5 minutes), followed by avidin/biotin block (Vector Laboratories, Burlingame, CA) (10 minutes each). Sections were incubated with the appropriate dilutions of primary rat anti-mouse mAbs against Foxp3 (eBioscience) and CCR9 (eBioscience), or isotype controls for 1 h, followed by incubation with a biotinylated secondary Ab for 30 min (mouse anti-rat IgG F(ab')<sub>2</sub>; Jackson ImmunoResearch Laboratories). Immunostaining was detected by incubating with streptavidin-HRP (DakoCytomation, Glostrup, Denmark) for 30 min and with diaminobenzidine-H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich, St.Louis, Mo) for 5-10 min. Slides were washed three times for 5 min each time in PBS between each incubation step, counterstained with hematoxylin, and mounted in aqueous mounting medium.

### **Statistical analysis**

Data are expressed as means  $\pm$  SD. Statistical analysis for significant differences was performed using Student's *t* test for unpaired data. *p* values of less than 0.05 were considered significant.



## RESULTS

### Administration of IMQ protects against TNBS-induced colitis

Previous studies demonstrated that TNBS-induced colitis is a Th1- and Th17-mediated condition with many features of CD<sup>1</sup>. By using the TNBS-induced murine colitis model, in this study, we investigated the efficacy of IMQ as an anti-inflammatory in both innate- and acquired-immunology-involved mucosal diseases. We initially evaluated the effective dose of IMQ and found that systemic administration of 10 µg of IMQ resulted in protection against TNBS-induced colitis. Mice treated with IMQ exhibited less body weight loss and significantly lower colon length shortening and colon weight (Figure 1A). Decreased disease severity was concordant with decreased histological score (Figure 1B). Histological examination showed fewer ulcerative lesions and less inflammatory cell infiltration in the colons of IMQ-treated mice when compared with saline-treated mice (Figure 1B).

In order to evaluate whether the protective effects of IMQ administration on TNBS-induced colitis are due to the cytokine balance induced by IMQ, we examined the levels of mRNA of several pro-inflammatory cytokines and anti-inflammatory cytokines in the affected colon on day 5 by qRT-PCR. As shown in Figure 2, administration of TNBS induced strong TNF- $\alpha$  mRNA expression in the colon and this expression was reduced in the IMQ-treated colon, suggesting that IMQ suppresses colonic inflammation. In contrast, we detected gene induction of IL-6 and IFN- $\beta$  in IMQ-treated mice, suggesting that IMQ activates TLR7 and produces IFN- $\beta$  and IL-6. Previous study showed that IL-6 doesn't increase in colon of TNBS-induced colitis<sup>25</sup>.

Therefore increased IL-6 expression in IMQ-treated mice was due to pDC stimulation by IMQ. We also detected strong induction of TGF- $\beta$ , which is thought to be anti-inflammatory cytokine in IMQ-treated colon, thus suggesting that IMQ also induces TGF- $\beta$ -producing cell, such as Tregs in the colon.

### **Treg proliferation after co-cultured with pDCs stimulated with IMQ**

It has been demonstrated that Foxp3<sup>+</sup> Tregs reduce the development of TNBS colitis<sup>26</sup>. Therefore, we next evaluated the potential effects of IMQ on induction Tregs *in vitro* using pDCs, as a key component in intestinal inflammation and colonic innate immune responses through TLR7 activation<sup>27, 28</sup>. Naïve CD4<sup>+</sup> T cells isolated from spleen were incubated with pDCs and stimulated with LPS as TLR4 agonist, IMQ, or CpG as a TLR9 agonist, respectively. As shown in Figure 3A, relatively low production of pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , and high production of the anti-inflammatory cytokine IL-10 was confirmed in IMQ-stimulated CD4<sup>+</sup>T cell supernatants. Increased IFN- $\gamma$  and TNF- $\alpha$  production after LPS stimulation might be suggesting cDC contamination in pDC. IL-4 as Th2 cytokine was not detected. To confirm that IMQ is able to induce IL-10-producing Treg proliferation, we analyzed the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs in the co-cultured T cells described above by flow cytometry. After comparing un-stimulated T cells with LPS stimulated T cells, we detected almost no differences in Treg proliferation after IMQ stimulation (Figure 3B). Overall, these findings indicate IMQ is not a direct inducer of Treg proliferation.

### **pDCs recruited into inflamed lymph nodes stimulate naïve T cells**

As pDCs are found in blood and secondary lymphoid organs, but not peripheral tissue in the steady state, we expected pDCs to be recruited in MLN and to stimulate naïve T cells. Therefore, we analyzed mRNA expression in the spleen and MLN of IMQ-treated mice. Although there was no marked mRNA expression in the spleen, strong expression of TLR7, IFN- $\beta$ , TGF- $\beta$  and Foxp3 mRNA was observed in MLNs (Figure 4A). In order to confirm Treg proliferation in MLNs, we measured cytokine production in MLN of TNBS colitis mice. As shown in Figure 4B, IFN- $\gamma$  (Th1 cytokine) and IL-17 (Th17 cytokine) were decreased in IMQ-treated mice. In contrast, IL-10 (anti-inflammatory cytokine) production was increased.

### **IMQ indirectly promotes accumulation of Tregs via induction of CCR9**

To confirm that Tregs were affected by the inflamed colon, we analyzed Treg expression in the colon by immunohistochemistry. As shown in Figure 5A, increased induction of Foxp3<sup>+</sup> Tregs was detected in IMQ-treated mouse colon. As we presumed that TLR7 activation is also able to induce CCR9<sup>+</sup> cells, we performed CCR9 immunohistochemistry in next. In IMQ-treated mouse MLNs, but not in colon, we detected CCR9<sup>+</sup> cell induction, as compared to untreated mouse MLNs and colon (Figure 5B).

## DISCUSSION

In this study, we have observed that intraperitoneal administration of IMQ can protect mice from TNBS-induced colitis. A previous study demonstrated that TLR7 agonist reduces joint inflammation in a mouse arthritis model<sup>29</sup>. Moreover, a TLR7 agonist retained anti-inflammatory properties in dextran sodium sulfate (DSS)-induced murine acute colitis and thioglycolate (TG)-elicited peritonitis models<sup>30</sup>. These models represent neutrophil-associated inflammation, and not specifically T cell-dependent inflammation. Several anti-tumor studies showed that IMQ therapy not only stimulates proinflammatory cytokines, but also stimulates the proliferation of Foxp3<sup>+</sup> regulatory T cells and IL-10 secreting CD4<sup>+</sup>T cells, both of which have been shown to be involved in self regulation of inflammation. Recent reports showed that defects in innate immunity, as well as reduced frequencies and suppressive functions of Tregs, are central to the pathogenesis of IBD<sup>31</sup>. Therefore, we selected TNBS-induced colitis to explore whether IMQ effects to both innate immune cells and T cells. TNBS-induced colitis is mediated by an immunological pathway involving Th1, Th17 and Tregs, which is comparable to the inflammation present in CD.

IMQ is a TLR7 agonist and a strong inducer of type I IFN. A previous study showed that type I IFN mediated by activation of TLR9 signaling pathway protects mice from experimental colitis<sup>15</sup>. Unlike TLR9 agonist, IMQ is commonly used in the treatment of genital warts and superficial basal cell carcinoma<sup>19</sup>. Thus, it seems reasonable to speculate that IMQ may also protect mouse colonic inflammation in the same manner as TLR9 agonist. In our study, TLR7 stimulation has less induced

pro-inflammatory cytokine such as TNF- $\alpha$  and IFN- $\gamma$  than TLR9 stimulation. Therefore, TLR7 agonist may be reasonable to use in inflammatory situation such as IBD. Although this raises the question of how type I IFN protects colonic inflammation.

In several studies of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), IFN- $\beta$  induction and signaling pathways play critical roles in suppressing Th17-associated inflammation via induction of IL-10 and IL-27 from macrophage and DCs<sup>32,33</sup>. This result is also correlated with clinical observations that IFN treatment leads to increased production of IL-10 in MS patients. In this study, we found that, in MLN of IMQ treated mice, strong expression of TLR7 and IFN- $\beta$  mRNA was observed, suggesting that pDCs activated with IMQ was recruited in MLNs. Furthermore, strong TGF- $\beta$  and Foxp3 mRNA expression suggested Treg induction in MLNs. We showed that IL-10 production was increased in MLNs of IMQ-treated mice, thus suggesting that IMQ administration is able to increase the Treg population and anti-inflammatory activity. IL-10 and TGF- $\beta$  are critical factors for Treg function, and are necessary to abrogate established intestinal inflammation, as demonstrated by the inability of Tregs to cure colitis if IL-10 signaling is blocked. We did not detect Treg proliferation *in vitro*, thus suggesting that pDC maturation in the appropriate environment was essential to stimulate naïve T cells. In contrast, an increased number of Tregs was observed on immunohistochemistry in the intestinal mucosa of IMQ-treated mice. These data suggest that pDCs in draining lymph nodes, such as MLN, mature with IMQ and stimulate naïve T cells to proliferate to Tregs. Finally, Tregs move to the site of inflammation in an attempt to halt the progression of colitis.

Previous study showed that type I IFN signaling ameliorates the severity of CD4<sup>+</sup> T-cell transfer model of colitis by increasing the number of Tregs<sup>34</sup>, and our result followed this observation. Although, the next question which is raised by our study is how Tregs are recruited to the inflamed colon.

CCR9 has been shown to be important in lymphocyte homing to the gut through interactions with its ligand CCL25<sup>35</sup>. In the context of intestinal inflammation, interactions between CCR9-expressing T cells and CCL25 expressed in the gut epithelium have been implicated as an important mechanism for recruiting circulating lymphocytes to the intestinal mucosa<sup>35</sup>. It was recently shown that CCR9-expressing pDCs are increased with TLR9 stimulation<sup>36</sup>. We showed that after TLR7 ligand IMQ administration CCR9-expressing cells are increased in MLN. This suggests that TLR7 activated by IMQ induces CCR9<sup>+</sup> cells, and that CCR9 induced Tregs, which were recruited to the inflamed intestine where they produced anti-inflammatory cytokines. It should be noted that a CCR9 antagonist has already been trialed in IBD treatment to regulate effector T cells migration into the intestine. Our data and other reports indicate the potential risk in IBD patients of blockade of effector T cells and Tregs in gut inflammation.

In conclusion, IMQ has been effectively and safely used to treat several diseases. Our results suggest that the beneficial effects of IMQ can be linked to accumulation of Tregs to intestinal inflammation. Therefore, we conclude that treatment with IMQ also has therapeutic potential also in IBD.

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**Competing interest statement**

The authors declare that they have no competing financial interests

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## Figure legends

**Figure 1.** Administration of IMQ ameliorates TNBS colitis.

A: Administration of IMQ significantly suppressed colonic inflammation in TNBS-induced colitis, as shown by body weight decreases, colon length and colon weight, as compared with saline-administered mice (n=8 in each group;  $p<0.05$ ).

B: Extensive superficial ulceration with mucosal inflammatory reactions, such as changes in colonic wall thickness and accumulation of inflammatory cells, induced by TNBS were abolished in mice treated with IMQ, as indicated by histological scores.

**Figure 2.** mRNA expression in colon of TNBS-administered mice treated with or without IMQ. TNF- $\alpha$  was decreased and strong expression of TGF- $\beta$  was detected in IMQ-treated colon, indicating that IMQ suppressed colonic inflammation. IL-6 and IFN- $\beta$  were strongly expressed after IMQ administration, thus suggesting that stimulated TLR7 may affect the anti-inflammatory effect of TNBS-induced colitis (n=8 in each group;  $p<0.05$ ).

**Figure 3.** Cytokine production and Treg induction of CD4<sup>+</sup> T cells co-cultured with pDCs, stimulated with LPS, IMQ or CpG.

A: Relatively lower production of pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , and high production of anti-inflammatory cytokine of IL-10 was observed in IMQ stimulated CD4<sup>+</sup> T cells (n=6 in each group;  $p<0.05$ ).

B: There were no differences in induction of Treg cells between naïve cells, LPS

stimulation and IMO stimulation *in vitro*.

**Figure 4.** A: mRNA expression in spleen and MLN of IMQ-treated mice was analyzed.

Although there was no marked mRNA expression in spleen, strong expressions of TLR7, IFN- $\beta$ , TGF- $\beta$  and FOXP3 mRNA were observed in MLN (n=8 in each group;  $p<0.05$ ).

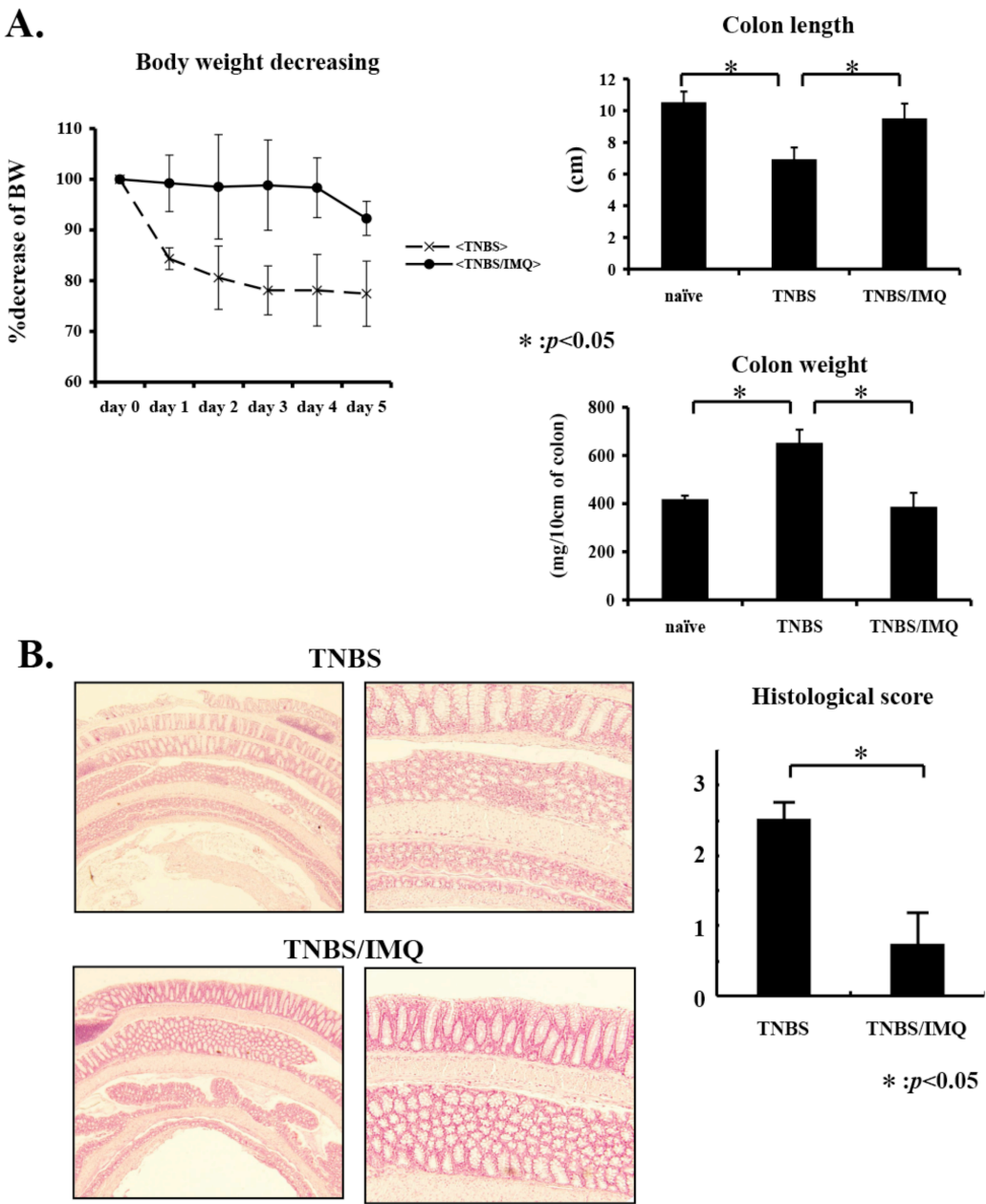
B: Cytokine production in MLN of TNBS colitis mice was measured. In IMQ-treated mice, IFN- $\gamma$  (Th1 cytokine) and IL-17 (Th17 cytokine) were decreased, while IL-10 (anti-inflammatory cytokine) production was increased (n=8 in each group;  $p<0.05$ ).

**Figure 5.** Tregs and CCR9 expression were analyzed in TNBS-induced mouse colon with or without IMQ treatment by immunohistochemistry (n=6 in each group;  $p<0.05$ ).

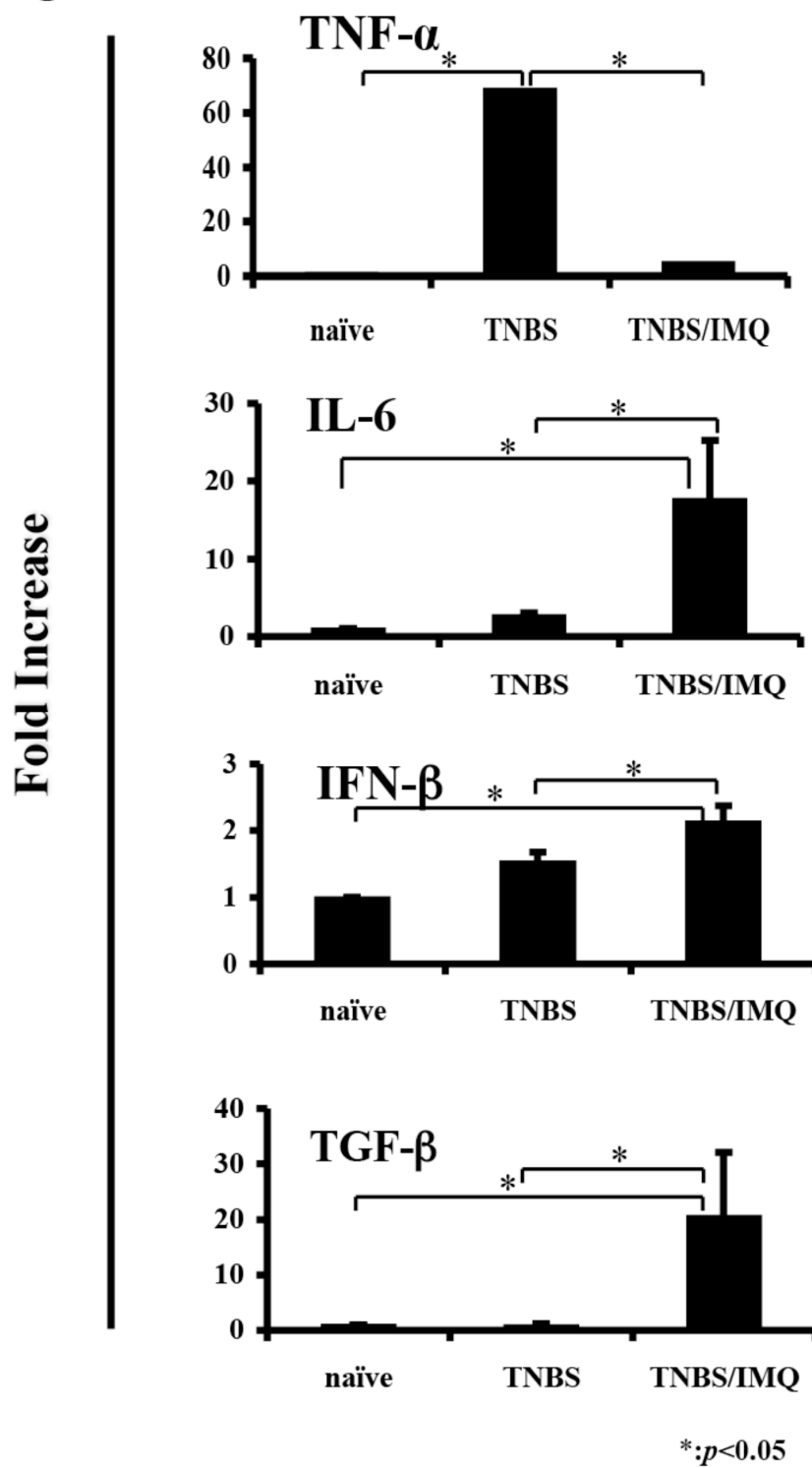
A: In IMQ-treated mouse colon, increased induction of Foxp3<sup>+</sup>Tregs was detected. Arrow indicates Foxp3<sup>+</sup>Tregs.

B: In IMQ-treated mouse MLN, but not in colon, increased induction of CCR9<sup>+</sup> cells was detected. Arrow indicates CCR9<sup>+</sup> cells.

Figure 1

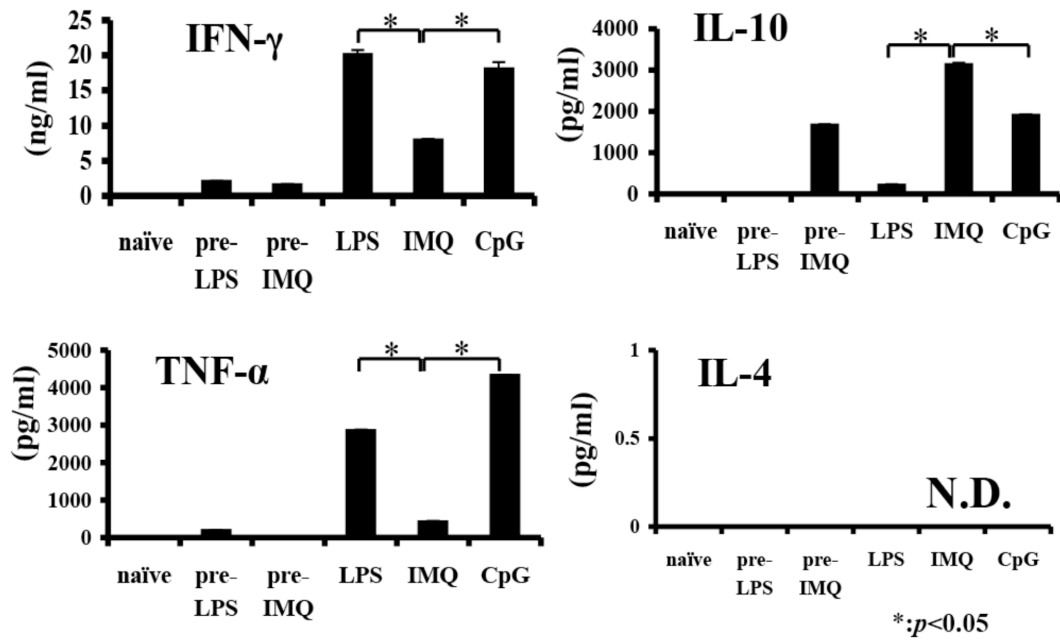


**Figure 2**

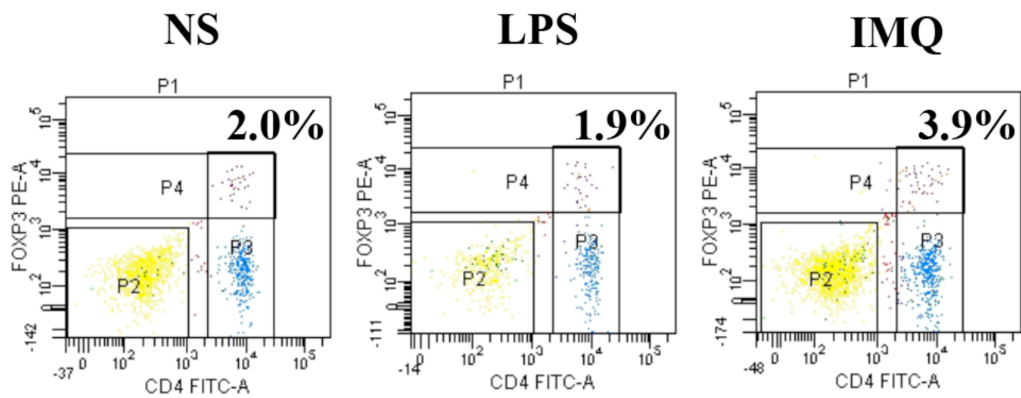


**Figure 3**

**A.**

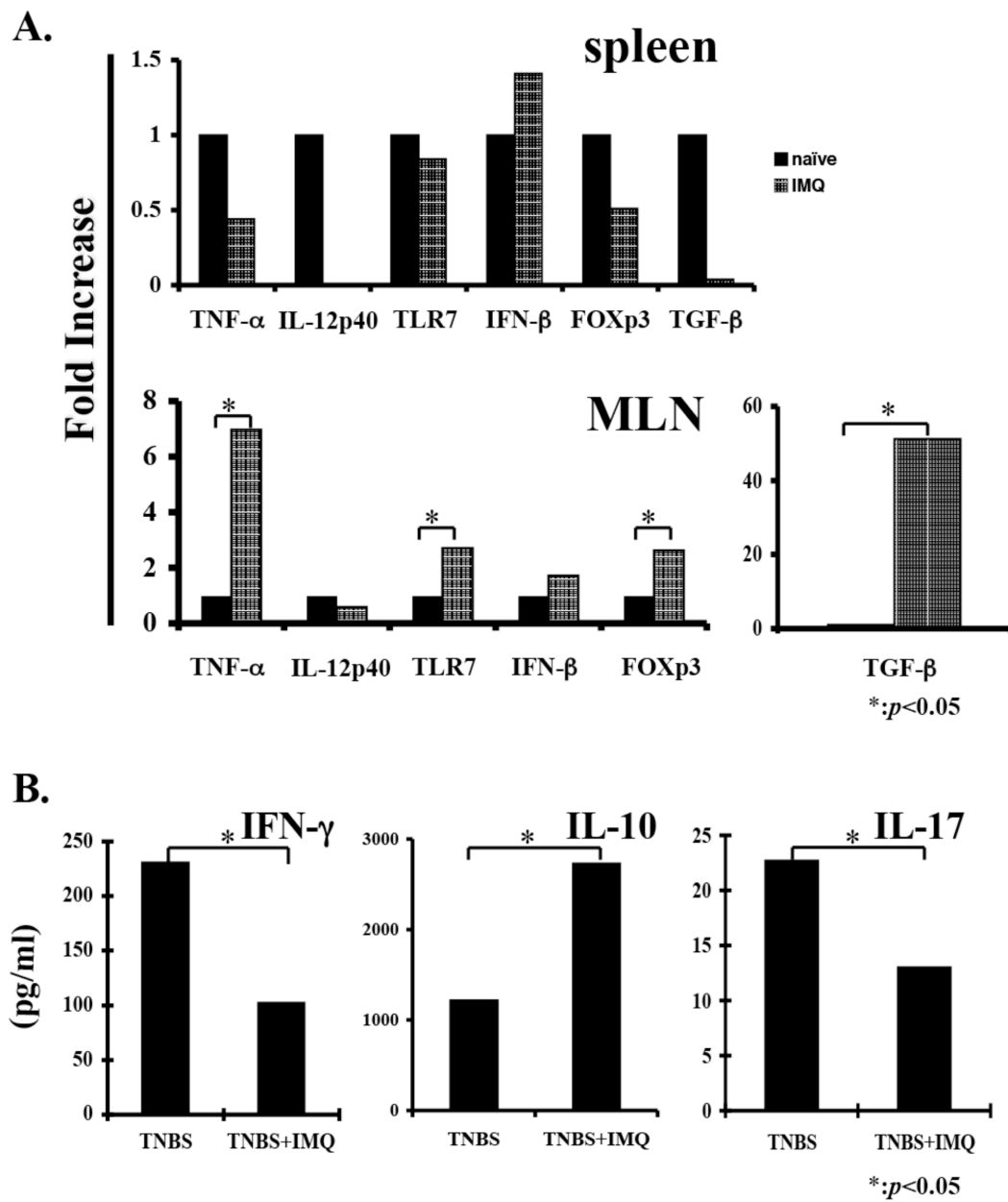


**B.**



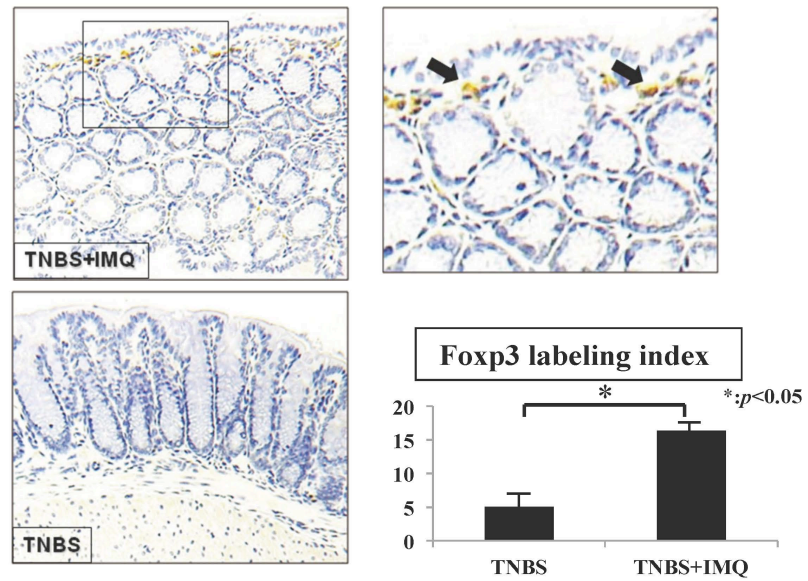


**Figure 4**



**Figure 5**

**A.**



**B.**

